Food and Chemical Toxicology 48 (2010) 2148–2153

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Rheosmin, a naturally occurring phenolic compound inhibits LPS-induced iNOS and COX-2 expression in RAW264.7 cells by blocking NF-κB activation pathway

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ARTICLE INFO

Article history:
Received 10 March 2010
Accepted 7 May 2010

Keywords:
Rheosmin
Pine needles
Inducible nitric oxide synthase (iNOS)
Inducible cyclooxygenase-2 (COX-2)
NF-κB activation

ABSTRACT

Inflammation is part of the host defense mechanism against harmful matters and injury; however, aberrant inflammation is associated to the development of chronic disease such as cancer. Raspberry ketone is a natural phenolic compound. It is used in perfumery, in cosmetics, and as a food additive to impart a fruity odor. In this study, we evaluated whether rheosmin, a phenolic compound isolated from pine needles regulates the expression of iNOS and COX-2 protein in LPS-stimulated RAW264.7 cells. Rheosmin dose-dependently inhibited NO and PGE_2 production and also blocked LPS-induced iNOS and COX-2 expression. Rheosmin potently inhibited the translocation of NF-κB p65 into the nucleus by IκB degradation following IκB-α phosphorylation. This result shows that rheosmin inhibits NF-κB activation. In conclusion, our results suggest that rheosmin inhibits LPS-induced iNOS and COX-2 expression in RAW264.7 cells by blocking NF-κB activation pathway.

1. Introduction

Inflammation is a complex process regulated by a cascade of cytokines, growth factors, nitric oxide (NO) and prostaglandins produced by activated macrophages. Macrophages are key players in the immune response to foreign invaders such as pro-inflammatory cytokines (Berenbaum, 2000). Activated macrophages play an important role in inflammatory diseases via production nitric oxide (NO) and prostaglandins (PGE_2) (Fujiwara and Kobaashi, 2005). Excess production of these inflammatory mediators is involved in many diseases including rheumatoid arthritis, atherosclerosis, asthma, pulmonary fibrosis and cancer (Bharat Reddy and Reddanna, 2009).

NO production is regulated by three isoforms of NO synthases (NOS). The neural (nNOS) and endothelial (eNOS) isoforms are constitutively expressed in select tissues, and their enzymatic activity is regulated by changes in the intracellular free Ca^{2+} concentration. A third member of the NOS family is inducible NOS (iNOS), which is produced in large quantities in response to inflammatory stimuli such as lipopolysaccharide (LPS), one of the most potent macrophage activators. NO is also involved in pathophysiological process such as inflammation and carcinogenesis (Moon et al., 2008). Therefore, the inhibition of NO over-production may be an important measure for evaluating the effects of anti-inflammatory drugs.

PGE_2 is an inflammatory mediator which is produced from the conversion of arachidonic acid by cyclooxygenase. In a variety of inflammatory cells, COX-2 is induced by cytokines and other activators, such as LPS, resulting in the release of a large amount PGE_2 at inflammatory sites (Yoon et al., 2009). COX-2 is undetectable in most normal tissues. It is an inducible enzyme, becoming abundant in activated macrophages and other cells at sites of inflammation. Therefore, the inhibition of COX-2 expression is believed to be the major target for assessing the anti-inflammatory drugs because COX-2 is usually specific to inflamed tissue.

Nuclear transcription factor kappa-B (NF-κB) regulates various genes involved in immune and acute phase inflammatory responses, and in cell survival (Li and Verma, 2002). NF-κB activation in response to pro-inflammatory stimuli involves the rapid phosphorylation of IκBs by the IKK signalosome complex (Karin and Delhase, 2000). The resulting free NF-κB then translocates to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes and induces the transcription of pro-inflammatory mediators such as iNOS and COX-2 (Baeuerle and Baltimore, 1996).

Rheosmin (Raspberry ketone) is a natural phenolic compound. It is used in perfumery, in cosmetics, and as a food additive to impart a fruity odor (Beekwilder et al., 2007). In the recent report, when given to mice in high doses (up to 2% of food intake), rheosmin has been shown to prevent high-fat-diet-induced elevations in body weight. This effect is reported to stem from the alteration of lipid metabolism, increasing norepinephrine-induced lipolysis. Although products containing this compound are marketed for weight loss, this effect has not been studied in humans (Morimoto et al., 2005). With the exception of this literature (Morimoto et al., 2005), however, other pharmacological effects of rheosmin have not yet been studied.

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doi:10.1016/j.fct.2010.05.020

In this study, we investigated the effect of rheosmin firstly isolated from pine needle (*Pinus densiflora*) on LPS-induced iNOS and COX-2 expression and clarified it mode of action in RAW264.7 cells. Rheosmin suppressed NF-κB activation by IκB-α degradation following IκB-α phosphorylation, which could regulate NO and PGE_2 related acute or chronic inflammation.

2. Materials and methods

2.1. Chemical reagents

LPS (*Escherichia coli* 055:B5) and 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Antibodies recognizing iNOS, COX-2 and p65 were purchased from Santa Cruze Biotechnology (Santa Cruz, CA, USA). IκB-α and phospho-IκB-α antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The secondary antibody, goat anti-rabbit IgG-HRP conjugated, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa 488, the secondary antibody for immuno-staining was purchased from Invitrogen (Carlsbad, CA, USA). Prostaglandin E_2 ELISA monoclonal Kit and Nuclear Extraction Kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). Dulbeco's modified Eagle's medium (DMEM), penicillin and fetal bovine serum (FBS) were purchased from Gibco Inc. (NY, USA). All electrophoresis chemicals were purchased from Bio-Rad Labs (Hercules, CA, USA).

2.2. Purification and identification of rheosmin from pine needles (P. densiflora)

Pine needles were randomly collected from 20 trees in Andong, Korea. One kilogram of pine needles was extracted with 3 L of 80% methanol with shaking for 24 h. After 24 h, the extract was filtered, concentrated to approximately 600 ml volume using a vacuum evaporator, and fractioned with *n*-hexane, chloroform and ethyl acetate in consecutive order with a separating funnel. Ethyl acetate fraction was collected, evaporated by a vacuum evaporator and kept at −80 °C for the purification of rheosmin with HPLC. Twenty micro liters of ethyl acetate fraction (4 mg/ml methanol) was injected into HPLC equipped with C_{18} column (DELTA PAK, 15 μm, 300A, 300 × 7.8 mm) equilibrated at ambient temperature and stabilized with the mobile phase {from a ratio of 1.6 to 8.4 (methanol:water) to a ratio of 5 to 5} at a flow rate of 2.5 ml/min for 40 min at the UV detector set at 280 nm. Eight fractions were collected with a fraction collector at regular intervals (5 min) and then stored in −20 °C for the identification. For identification, purified five fractions were analyzed by GC–MSD, equipped with a Supelcowax 10 fused silica capillary (30 m length × 0.25 mm i.d. supelco, USA). Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. Two micro liters of fractions was injected into the column using 10:1 of the split ratio injection mode. The oven temperature was initially held at 100 °C for 3 min, then raised to 300 °C in 5 min, and finally held at 300 °C for 48 min. The temperatures of injector and detector were 200 °C and 240 °C, respectively. The mass detector was operated in electron impact mode with an ionization energy of 70 eV, a scanning range of 33–550 a.m.u. and a scan rate of 1.4 scans/s. purified rheosmin was positively identified by comparing the mass spectra and RIs in the Wiley 275 Imass spectral database (Hewlett–Packard, 1995).

2.3. Cell culture

The murine macrophage cell line RAW264.7 was purchased from the Korean Cell Line Bank (Seoul, Korea). RAW264.7 cells were cultured in Dulbeco's modified Eagle's medium (DMEM) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum. The cells were incubated in an atmosphere of 5% CO_2 at 37 °C and subcultured every 2 days. In all experiments, cells were grown to 80–90% confluence and subjected to no more than 20 cell passages.

2.4. MTT assay

To evaluate the effect of rheosmin on cell viability, RAW264.7 cells were plated in 96-well plates at a density 5×10^4 cells/well for 24 h. The cells were treated with the varying concentrations of rheosmin (125, 250, 500 and 1000 μg/ml) for 24 h at 37 °C. Fifty micro liters of MTT solution (1 mg/ml, St. Louis, MO) to each well and cells were further incubated for 4 h at 37 °C. Media were discarded and 100 μl of DMSO was added to each well for the solubilization of formazan. Optical density was measured at 450 nm on a SepectraMax 340 microplate reader.

2.5. Measurement of NO and PGE_2

Inhibitory effects of rheosmin on the production of NO in RAW 264.7 cells was evaluated using a method modified from that previously reported (Banskota et al., 2003). RAW 264.7 cells (2×10^5 cells/well) in 10% FBS-DMEM without phenol red were seeded in a 6-well plate for 24 h at 37 °C. Cells were washed with 1 × PBS, replaced with fresh media, and then treated with the varying concentrations of rheosmin (125, 250, 500 and 1000 μg/ml) for 1 h. LPS (1 μg/ml; Sigma–Aldrich, Youngin, Korea) was treated for 24 h at 37 °C. After 24 h, 200 μl of the medium were placed in a 96-well plate and an equal amount of Griess reagent (1% sulfanilamide and 0.1% N-1-(naphthyl) ethylenediamine-diHCl in 2.5% H_3PO_4) was added. The plate was incubated for additional 5 min at the room temperature and then the absorbance was measured at 540 nm with a SepectraMax 340 microplate reader. The amount of nitric oxide was calculated using sodium nitrite standard curve. For the measurement of Prostaglandin E_2 (PGE_2) production, 50 μl of the supernatant of cultured medium was collected, and PGE_2 production was determined using prostaglandin E_2 ELISA monoclonal Kit with manufacturer's instructions.

2.6. SDS–PAGE and Western Blot analysis

RAW 264.7 cells (2×10^5 cells/well) were seeded in a 6-well plate for 24 h at 37 °C. Cells were washed with 1 × PBS, replaced with fresh media, and then treated with the varying concentrations of rheosmin for 1 h. LPS (1 μg/ml) was treated for 24 h or 30 min. The cells were washed and scraped into cold phosphate-buffered saline (PBS) and centrifuged at 500*g* at 4 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM DTT) containing 1% Triton X-100 for 30 min at 4 °C and centrifuged to yield whole-cell lysates. Cytosol and nuclear protein were extracted with Nuclear Extraction Kit. The proteins (50 μg) were separated by 8% (iNOS and COX-2 protein) or 15% (IκB-α, phospho-IκB-α and NF-κB p65) sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred into PVDF membranes. The PVDF membranes were then blocked for non-specific binding in blocking buffer (5% non-fat milk in 1 × TBS solution containing 0.1% Tween-20) for 1 h and then washed with 1 × TBS solution (0.1% Tween-20 in 1 × TBS). Subsequently, the membranes were incubated with iNOS and COX-2 antibody at 1:1000 dilutions in antibody dilution buffer (3% non-fat milk in 1 × TBS containing 0.1% Tween-20) with gentle shaking at 4 °C for 18 h and then washed with 1 × TBS. For IκB-α, phospho-IκB-α and p65, the membranes were incubated with IκB-α, phospho-IκB-α and p65 antibody at 1:500 dilutions in antibody dilution buffer (5% BSA in 1 × TBS containing 0.1% Tween-20) with gentle shaking at 4 °C for 18 h and then washed with 1 × TBS. After washing, the membrane was incubated with Phototope-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked antibody as the secondary antibody at 1:1000 dilutions in antibody dilution buffer (5% non-fat milk in 1 × TBS solution containing 0.1% Tween-20) for 1 h at the room temperature and then washed again. After washing, the membranes were treated with the detection agent (Amersham Biosciences) and immediately developed in Polaroid film.

2.7. Immuno-staining for translocation of p65

RAW 264.7 cells (2×10^5 cells/well) were cultured in 6-well plates containing a cover glass for 24 h at 37 °C. Cells were washed with 1 × PBS, replaced with fresh media, and then treated with the varying concentrations of Rhosmin for 1 h. After 1 h, LPS (1 μg/ml) was treated for 30 min at 37 °C. After 30 min, the cells were fixed with 2% formaldehyde for 30 min and then washed with 1 × PBS for 5 min three times. PBS with 10% fetal bovine serum (PBS/FBS) was then added to block non-specific binding for an hour. After washing, p65 polyclonal antibody was diluted in 0.1% saponin/PBS/FBS solution at 1:500 dilutions with gentle shaking at 4 °C for 18 h, and then cells were washed with 1 × PBS for 5 min three times. Subsequent antibody, Alexa-Flour 488 goat anti-rabbit IgG, and DAPI were used against p65 antibody and nuclei, respectively, and the cells were incubated in the dark for 1 h. Then, the cells were washed with 1 × PBS and mounted with antifade mounting medium. Mounted slides were viewed under a fluorescence microscope using a 60 × oil immersion objective. The excitation wavelengths for DAPI and p65 were 359 and 494 nm, respectively.

2.8. Statistical analysis

All results were expressed as the mean ± the standard deviation of triplicate analysis. Statistical comparisons were performed using the Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Identification of rheosmin from pine needles

Pine tree (*P. densiflora*) contains many functional compounds such as alpha-pinene, beta-pinene, fenchone, abietic acid, pimaric acid, camphene, flavonoid, quercetin, kaempferol and neoabietic acid. However, it has not yet been reported that pine tree contains rheosmin, a naturally occurring phenolic compound. In our study, rheosmin was firstly isolated and identified from pine needle. Fig. 1A and B shows the chromatogram and mass spectrum of purified rheosmin. Mass spectrum of purified rheosmin showed a